Remarks

Claims 1-5, 7, 10, 34-38, and 40-47 are pending in this application. Claims 1-5, 7, 10, 34-38, and 40-47 now stand rejected.

Claims 6, 8-9, 11-34, and 39 have been cancelled. Applicants reserve the right to pursue the cancelled claims in a continuing application.

New claims 48-63 are presented for Examination.

Claim Rejections – 35 U.S.C. § 103

Claims 1-3, 5, 7, 10, 34-36, 38, and 40 remain rejected, and claims 41-44 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Hoon et al. (US Patent 6,057,105) in view of Scholl et al. (2/01, Cancer Research, 61:823-826) for the reasons stated in the Office Action of 11/4/09 and for the reasons set forth below.

Claims 1-5, 7, 10, 34-38, and 40 remain rejected and newly added claims 41-47 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Hoon et al. (US Patent 6,057,105) in view of Scholl et al. (2/01, Cancer Research, 61:823-826) as applied to claims 1-3, 5, 7, 10, 34-36, 38, and 40 above, and further in view of Johansson et al. (2000, Clinical Chemistry, 46(7): 921-927) for the reasons stated in the Office Action of 11/4/09 and for the reasons set forth below.

Applicants respectfully traverse the present rejections for the following reasons. Both of the pending rejections are addressed together. Applicants have previously provided evidence of the unexpected results provided by the claimed panel of gene markers. In support of these results, Applicants now show that the combination of MAGE-A3[1], GalNAcT, MART-1, and PAX3 provided spectacularly unexpected results in predicting

[1] MAGE-A3 is the same as MAGE-3.

melanoma prognosis. Applicants will further show that the unexpected results are significantly due to the incorporation of GalNact and PAX3 into the panel of gene markers.

Claims 2, 36-40, 43, and 45-47 are directed to a method of predicting melanoma prognosis using the combination of MAGE-A3, MART-1, GalNAcT, and PAX3 marker. This combination provides a remarkable segregation of patient prognosis with respect to the number of markers expressed in a histologically negative sentinel lymph node from a melanoma patient. (see Survival Plots attached as Exhibit A). In particular the disease free survival results of the present invention are found to be exceedingly superior to the published disease free survival (DFS) results for the combination MAGE-A3, MART-1, Tyrosinase, and GP100. (Scoggins et al. attached as Exhibit B). The following disease free survival graphs are for the present invention:

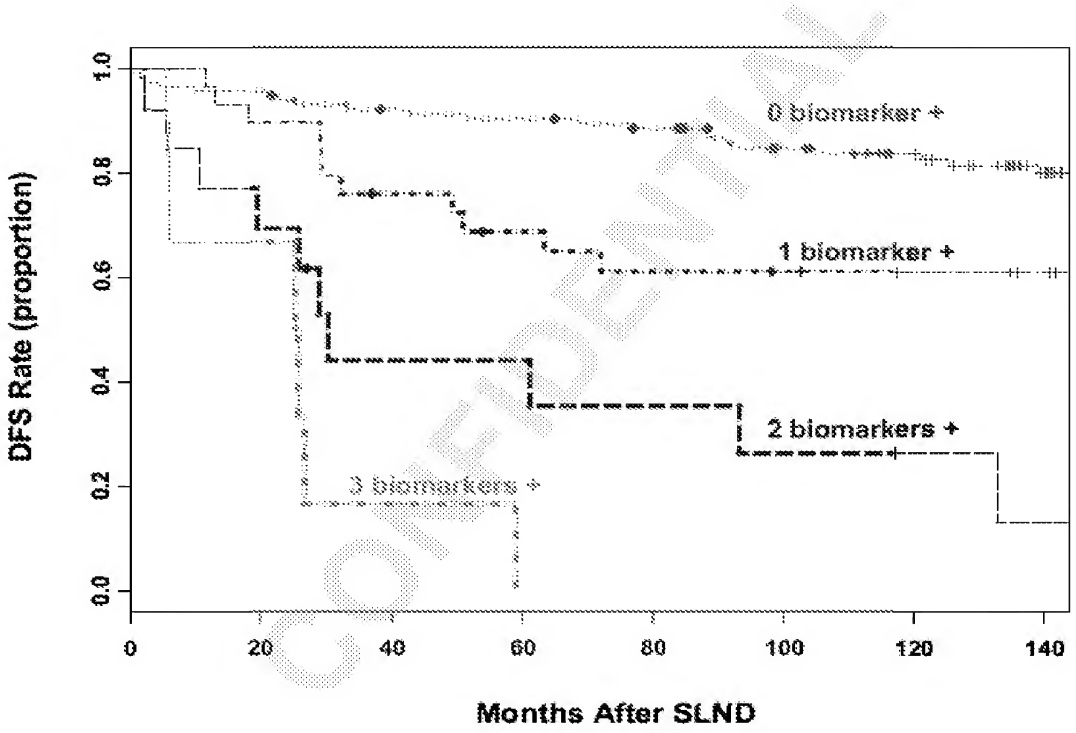
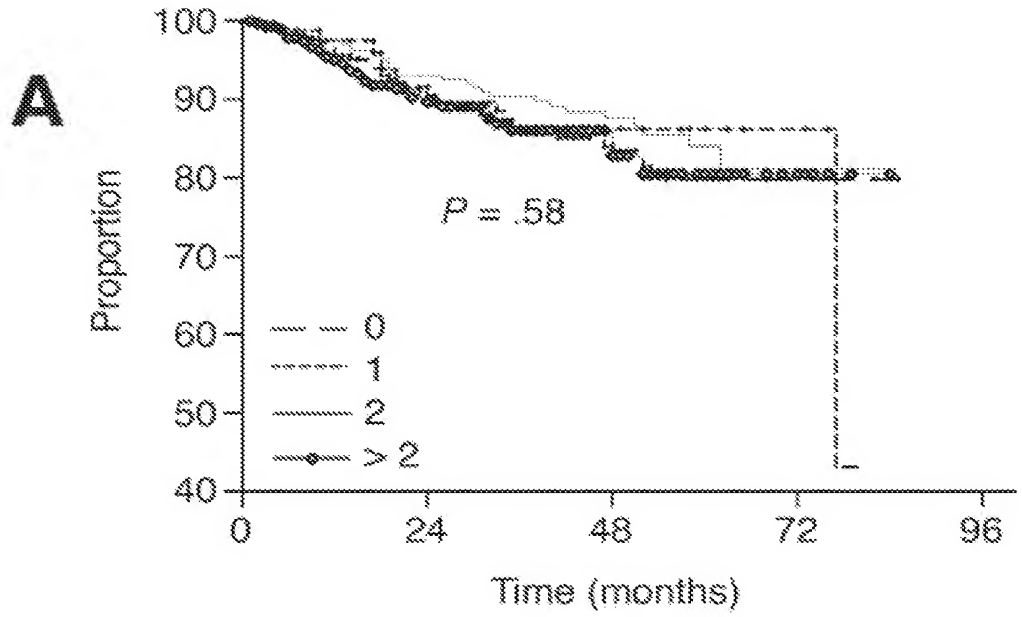


Figure 2A from Scoggins et al. provides the following DFS for the panel consisting of MAGE-A3, MART-1, Tyrosinase, and GP100:



It is quite evident by comparison of these two figures that the combination of markers from the present invention shows a readily observable segregation of disease free survival as a function of the number of positive markers. This is in stark contrast to the Scoggins reference which provides no prognostic value with respect to segregation of survival by the number of positive markers. The marker panels of the present invention and the Scoggins reference both include MAGE-A3, MART-1. The present invention further includes GalNact and PAX3 while the Scoggins reference further includes Tyrosinase, and GP100. Although disease free survival is an important indicator for accessing prognosis specific to disease progression and reoccurrence in its own right, Exhibit A also provides distance disease free survival (DDFS), and overall survival rate (OSR). In each of these additional survival results, the present invention significantly exceeds the results of Scoggins (Exhibit B, Figures 2B and 2C). It should be noted that the ordinate in the Scoggins figures is expanded thereby spreading the results out. Exhibit C provides a comparison of the Scoggins survival data and the present invention with the scales adjusted to be the same. It is an inescapable conclusion that the superior results of the present invention are due to the incorporation of GalNact and PAX3 into the marker panel.

This prognostic power of the present invention is even more remarkable in light of the published results in Hilari et al. regarding the individual ability of GalNact and PAX3 expression in discriminating between positive and benign lymph nodes in melanoma patients. (Hilari et al., attached as Exhibit C). Hilari et al. states the following:

> **Also, no difference was seen between positive and benign nodes for PAX3 and GalNAc-T when using our primer design.** However, when using the Takeuchi et al. primers and probes to analyze the same samples we found that PAX3 (but not GalNAc-T) did indeed show discriminatory power between benign versus positive nodes and to a lesser degree between positive nodes versus benign nevi (Fig. 2d). Therefore, we further tested the Takeuchi design for PAX3 on a subset of sentinel nodes from patients in our cohort. Fifty-seven nodes from 38 patients (28 without recurrence and 10 out of 11 patients that recurred) were selected for this analysis to determine if PAX3 had any prognostic potential in our cohort. **Figure 3 shows that there was no difference in PAX3 expression in nodes from patients with and without disease recurrence and therefore no further samples were tested**.
>
> Hilari et al., p. 180 (emphasis added)

It is clear that GalNact alone has practically no predictive value in discriminating positive and cancer free lymph nodes. The predictive value of PAX3 expression is, at best, marginal.

The combination of two markers from a non-predictive panel (MAGE-A3, MART-1), a non-discriminating marker (GalNact), and a marginally discriminating marker (PAX3) do not logically sum to the DFS results of the present invention. The Hilari et al. results are consistent with Dr. Pihan's analysis regarding Scholl et al. in which he states:

> The Scholl reference does not teach a method of detecting melanoma. Moreover, one skilled in the art of pathology or molecular biology would not interpret the teachings of the Scholl reference as suggesting that the detection of PAX3 in histopathologically negative lymph nodes would be functional as a test for occult melanoma metastasis.

(Amendment dated March 23, 2010, Pihan Declaration)

Applicants do not desire to misuse the word "synergistic," but it is quite clear from the evidence that the whole is much greater than the sum of the parts with respect to the combination of MAGE-A3, MART-1, GalNAcT, and PAX3.

Independent claims 1 and 35 are directed to a method of predicting melanoma prognosis using the combination of GalNAcT and PAX3 markers. Applicants have shown that GalNact and PAX3 are capable of conferring remarkable prognostic power to an otherwise unremarkable combination of markers. Therefore, for at least this reason, claims 1 and 35, along with their respective dependent claims, are allowable over the present rejections.

Independent claim 41 is directed to a method of predicting melanoma prognosis using the PAX3 marker. With respect to claim 41 and its dependent claims 42-44, Applicants rely on the evidence and arguments previously of record.

Finally, with respect to all pending claims, Applicants also continue to rely on the evidence and arguments previously of record.

Accordingly, for at least these reasons, claims 1-5, 7, 10, 33-38, 40-47, and 48-63 are allowable under the present rejections.

Conclusion

Applicants have made a genuine effort to respond to each of the Examiner's objections and rejections in advancing the prosecution of this case. Applicants believe that all formal and substantive requirements for patentability have been met and that this case is in condition for allowance, which action is respectfully requested. If any additional issues need

to be resolved, the Examiner is invited to contact the undersigned at his earliest convenience.

Please charge any fees or credit any overpayments as a result of the filing of this paper to our Deposit Account No. 02-3978.

Respectfully submitted,

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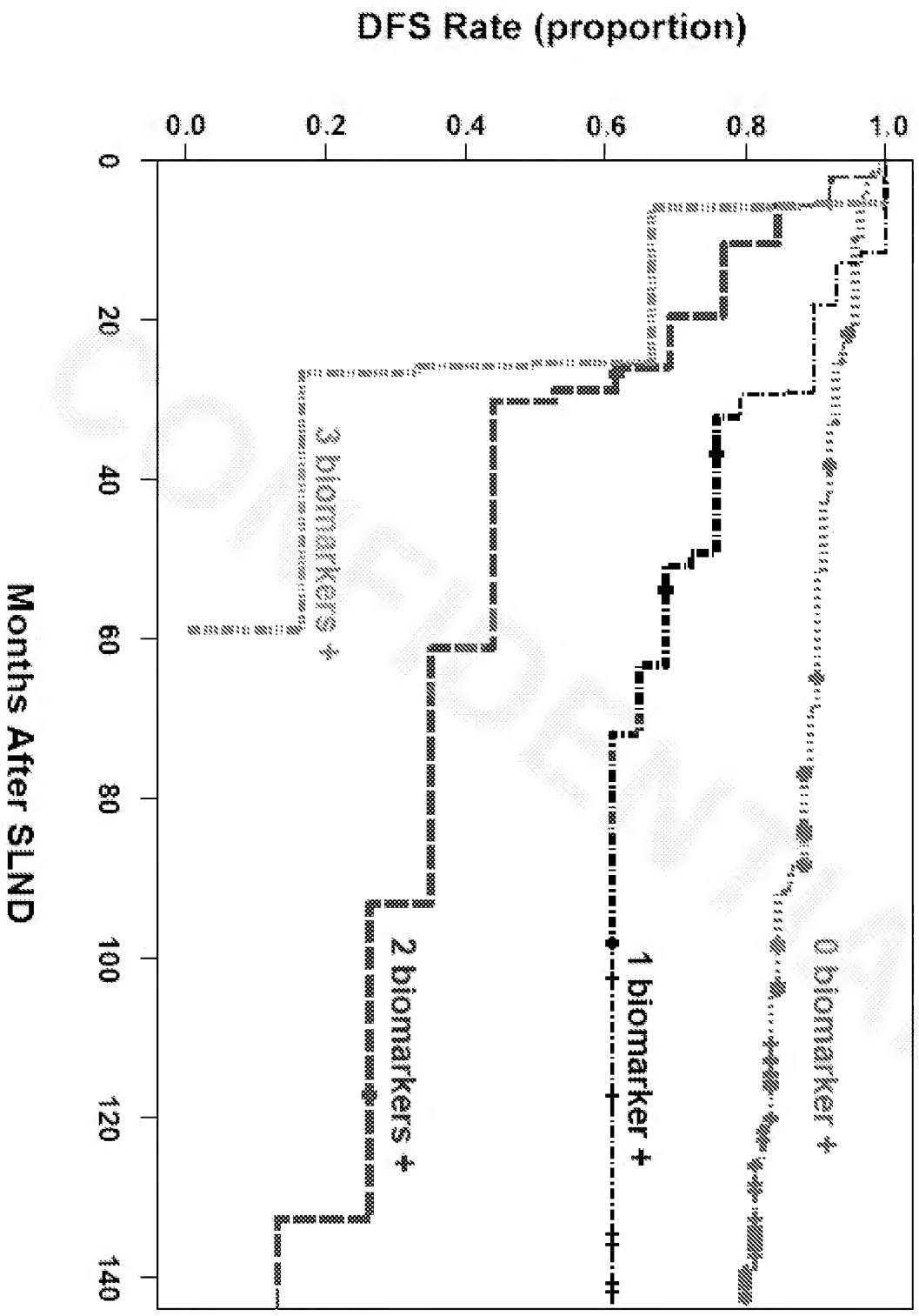
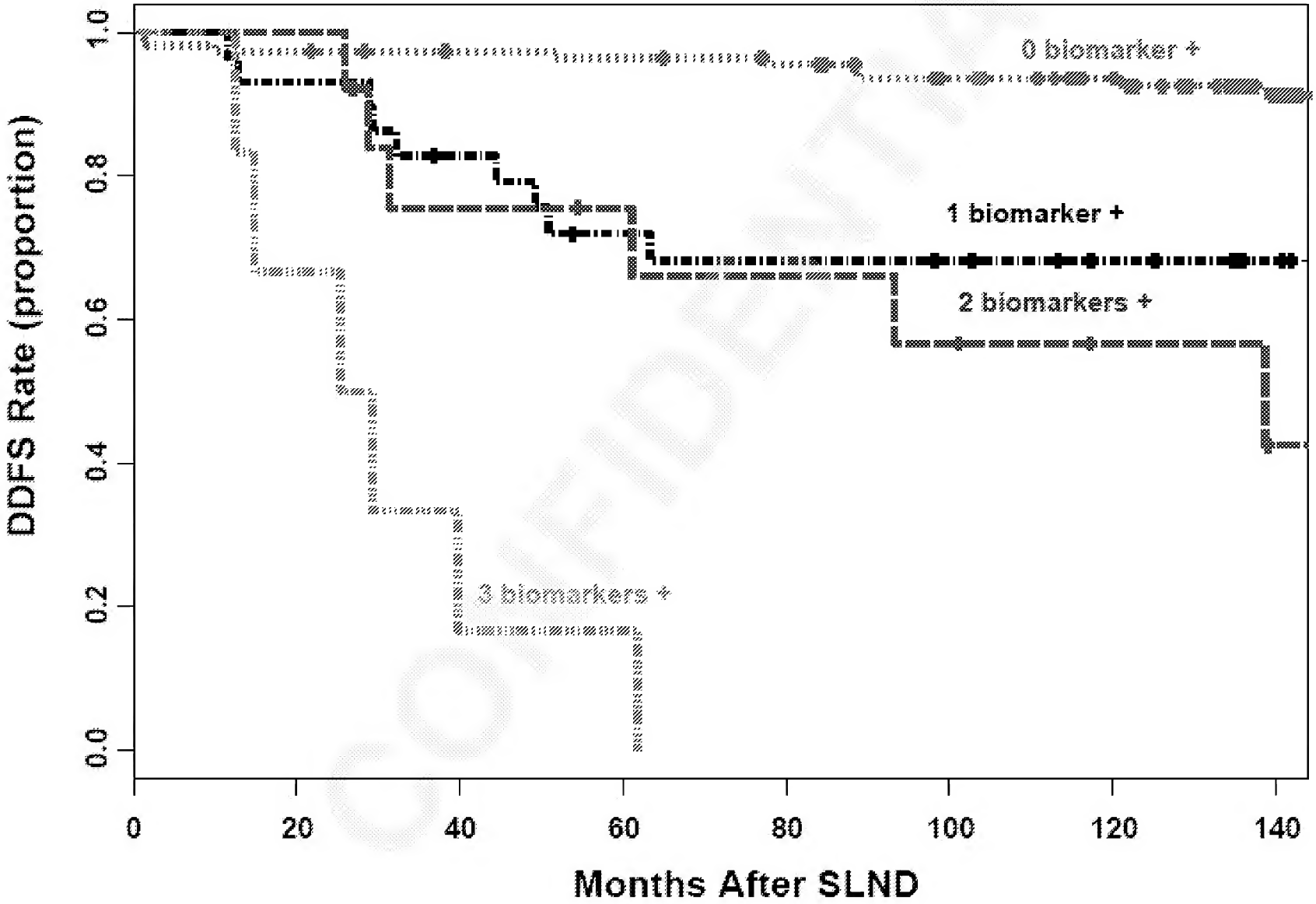
EXHIBIT A

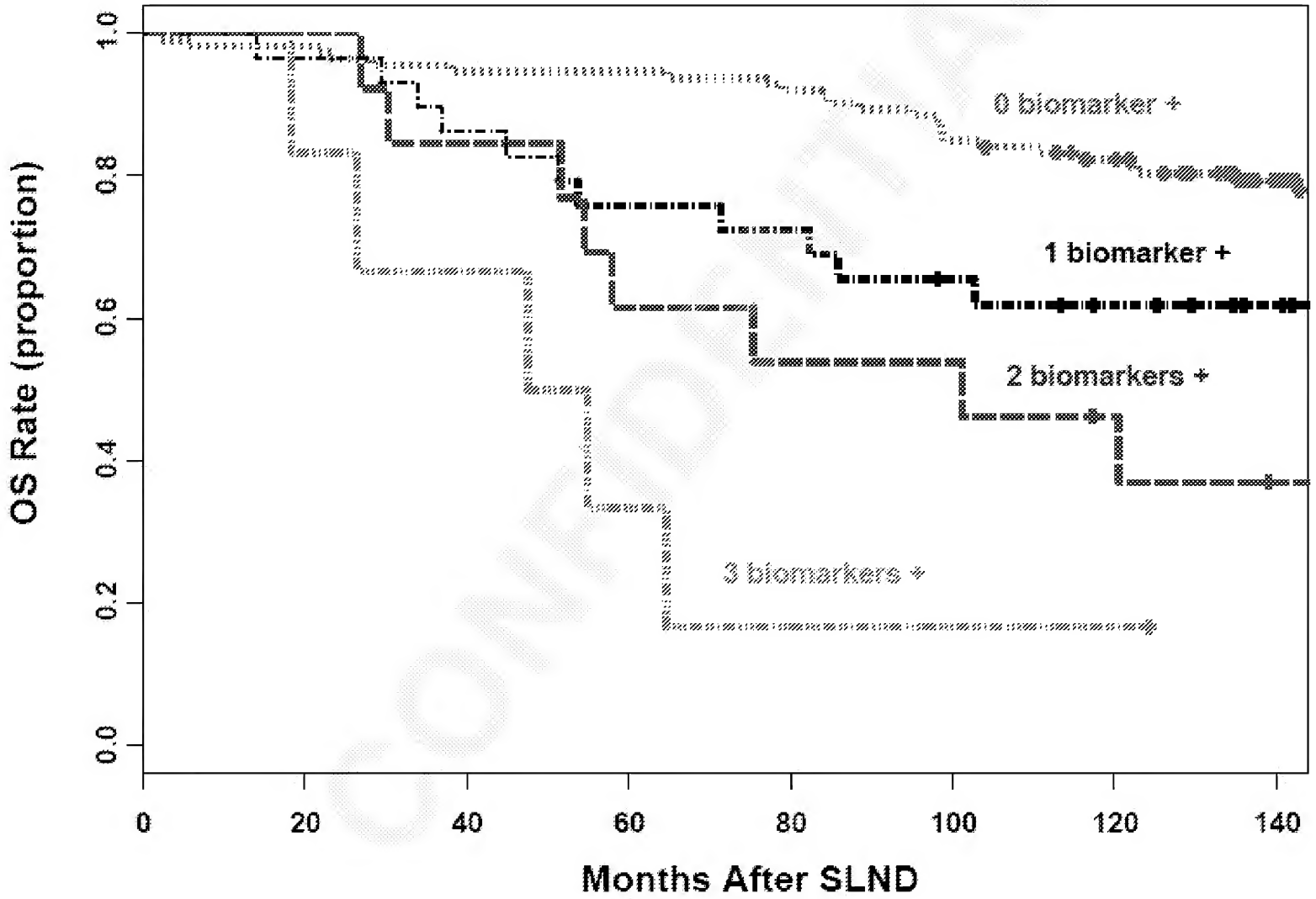


Present Invention

Present Invention



Present Invention



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ORIGINAL REPORT

Prospective Multi-Institutional Study of Reverse Transcriptase Polymerase Chain Reaction for Molecular Staging of Melanoma

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/06/2418-2849/$20.00

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ABSTRACT

Purpose
To evaluate the prognostic significance of molecular staging using reverse transcriptase polymerase chain reaction (RT-PCR) in detecting occult melanoma cells in sentinel lymph nodes (SLNs) and circulating bloodstream.

Patients and Methods
In this multicenter study, eligibility criteria included patient age 18 to 71 years, invasive melanoma ≥ 1.0 mm Breslow thickness, and no clinical evidence of metastasis. SLN biopsy and wide excision of the primary tumor were performed. SLNs were examined by serial-section histopathology and S-100 immunohistochemistry. A portion of each SLN was frozen for RT-PCR. In addition, RT-PCR was performed on peripheral-blood mononuclear cells (PBMCs). RT-PCR analysis was performed using four markers: tyrosinase, MART1, MAGE3, and GP-100. Disease-free survival (DFS), distant–DFS (DDFS), and overall survival (OS) were analyzed.

Results
A total of 1,446 patients with histologically negative SLNs underwent RT-PCR analysis. At a median follow-up of 30 months, there was no difference in DFS, DDFS, or OS between the RT-PCR–positive (n = 620) and RT-PCR–negative (n = 826) patients. Analysis of PBMC from 820 patients revealed significant differences in DFS and DDFS, but not OS, for patients with detection of more than one RT-PCR marker in peripheral blood.

Conclusion
In this large, prospective, multi-institutional study, RT-PCR analysis on SLNs and PBMCs provides no additional prognostic information beyond standard histopathologic analysis of SLNs. Detection of more than one marker in PBMC is associated with a worse prognosis. RT-PCR remains investigational and should not be used to direct adjuvant therapy at this time.

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INTRODUCTION

The most important predictor of survival for patients with early-stage melanoma is the regional nodal status.[1] Modern management of melanoma includes sentinel lymph node (SLN) biopsy. The SLN is the first node that receives lymphatic drainage from the primary tumor; therefore, it reflects the status of the entire basin.[2-5] SLN biopsy represents a minimally invasive method of nodal staging.

Despite the accuracy of SLN biopsy in documenting nodal status, a significant number of SLN-negative patients (approximately 10% to 20%) will develop metastatic disease.[6-9] Attempts to identify the stage I and II patients at greatest risk for recurrence have focused mainly on detection of melanoma-specific mRNA using reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is a highly sensitive technique that can detect a single melanoma cell within 1 million normal cells.[10,11]

RT-PCR for metastatic melanoma has been studied in various tissue types, including SLNs[12-15] and blood.[12,16,17] The tyrosinase gene has been most commonly evaluated by RT-PCR in melanoma research.[12-15,18-21] Other markers, such as MART1,[13,14,18,20,22] MAGE3,[14,18] and others,[14,20] have been studied. Based on these data, we sought to evaluate the prognostic significance

of RT-PCR of SLNs and peripheral-blood mononuclear cells (PBMCs) to detect melanoma patients at greatest risk of recurrence and mortality.

PATIENTS AND METHODS

The Sunbelt Melanoma Trial

The Sunbelt Melanoma Trial is a prospective, randomized trial involving 79 institutions that is evaluating the role of RT-PCR for ultrastaging, lymphadenectomy, and adjuvant interferon alfa-2b for patients with early nodal metastasis.[23] This study was approved by the institutional review board of each institution. Patients age 18 to 71 years with invasive melanoma $\geq$ 1 mm Breslow thickness and without clinical evidence of regional or distant metastasis were eligible.

Random assignment was accomplished using a randomized permuted block with stratification by tumor thickness and ulceration, with reassignment to ensure that each center was roughly balanced as to the number of patients in each arm. Following Zelen,[24] reassignment was done if the difference in treatment group sample sizes was more than a small randomly chosen positive integer. Power and sample size calculations were based on a comparison of survival time between the treatment arms with the following assumptions: 5-year accrual time, 10-year minimum follow-up time (for therapeutic results), right random censoring, one-sided significance level of .05, and a power level of at least 80% for detecting a 10% change in overall survival.

After informed consent was provided, patients underwent excision of the primary melanoma and SLN biopsy using intradermal injection of technetium sulfur colloid around the primary tumor site. A lymphoscintigram was obtained and a hand-held gamma probe was used intraoperatively to guide SLN identification. Intradermal injection of isosulfan blue dye (1 to 5 mL) was performed in the majority of patients as well. All blue nodes and all nodes $\geq$ 10% of the most radioactive or hottest node were collected as SLNs.[25]

A portion of each SLN (defined as one fourth of the lymph node or a 2-mm^3 portion of the node, whichever was smaller) was snap-frozen on dry ice or liquid nitrogen and stored at −80°C until it was shipped on dry ice. If more than one SLN was found, each SLN was processed identically. The remaining SLN tissue was processed by hematoxylin and eosin (H and E) staining at multiple levels, with at least five sections per block, along with two additional

Table 1. Clinical and Pathological Characteristics of the Patient Population With Regard to SLN RT-PCR Results

Characteristic	SLN RT-PCR Combined (RT-PCR positive, negative) (n = 1,446 patients)						
			Positive RT-PCR		Negative RT-PCR		
	No.	%	No.	%	No.	%	P*
Sex							.32
Male	807	56	335	55	472	58	
Female	621	43	274	45	347	42	
Unlisted	18	1					
Age, years							.12
Median	51						
Range	18-71						
Site of primary tumor							.06
Trunk	592	41	269	44	323	39	
Extremity	651	45	272	45	379	46	
Head or neck	185	13	66	11	119	14	
Other	2	< 1	2	< 1	0	0	
Unlisted	16						
Breslow thickness (mm)							.25
Median	1.6						
Mean	2.1						
Clark level							.47
I	5	< 1	3	< 1	2	< 1	
II	23	2	2	< 1	21	3	
III	356	25	156	26	200	25	
IV	992	69	427	71	565	70	
V	32	2	13	2	19	2	
Unknown	38						
Ulceration							.73
Present	336	23	145	24	191	23	
Absent	1,088	75	463	76	625	76	
NA	7	< 1	4	1	3	< 1	
Unknown	15	1					
SLN positive by H and E and/or IHC	0	0					
RT-PCR results†							
Negative	826	57					< .0001
Positive	620	43					

Abbreviations: SLN, sentinel lymph node; RT-PCR, reverse transcriptase polymerase chain reaction; NA, not available; H and E, hematoxylin and eosin; IHC, immunohistochemistry.

*For continuous variables, *P* value for testing for a difference in means for RT-PCR results. For categorical variables, *P* value for χ^2 test for association with RT-PCR results, omitting unknown categories. For the RT-PCR results, the *P* value compares the two proportions.

†RT-PCR results are given for SLNs, respectively, using the a priori protocol definition of a positive RT-PCR test.

random sections for S-100 immunohistochemistry (IHC). A histologically positive SLN was defined as evidence of metastatic tumor cells identified by either H and E or IHC. A central pathology review committee evaluated the first 10 patients from each participating institution, as well as all samples of SLNs containing metastases.

Patients also had blood drawn at the time of consent, 3 months postoperatively, and annually thereafter. As a result of logistic issues related to processing blood specimens, several sites were granted exemptions from drawing the blood samples, resulting in fewer blood samples for RT-PCR testing. The first tube of blood drawn was discarded to diminish the chance of contamination from the skin plug generated during venipuncture. After collection of 5 mL of blood in EDTA-containing tubes, peripheral blood mononuclear cells (PBMCs) were isolated with a hypotonic density gradient solution (DOT kit, National Genetics Institute, Los Angeles, CA) as described previously,[26] and total RNA was shipped to the central laboratory on dry ice.

SLNs and PBMCs were processed and analyzed by a central laboratory (National Genetics Institute) that was blinded to clinical and pathologic data. Total RNA was extracted using TRIREAGENT (Molecular Research Center Inc, Cincinnati, OH), quantified by absorbance at 260 nm, then precipitated with ethanol.[27] The quality of the RNA isolated was demonstrated by amplification and detection alongside beta-actin. RT-PCR was performed with specific primers for tyrosinase, MART1, MAGE3, and GP-100, followed by Southern blot detection.[26-28] The Southern blot signals were analyzed by determining optical band density for samples and controls. Test samples with optical densities more than 50% that of the negative controls were considered positive. Negative controls for SLNs included RNA from negative nodes as well as RNA from human melanoma cell lines. PBMC were collected from healthy controls and RNA from these negative controls was tested side by side with patient samples.

The a priori definition of a positive SLN RT-PCR test was detection of tyrosinase mRNA plus at least one other marker. Using this definition, we had no false-positive results when 100 nodes from patients without melanoma were analyzed during initial assay validation studies.[29] RT-PCR for PBMCs was considered positive if any marker was detected at any point in time. The study population for RT-PCR testing of SLNs included patients who were histologically negative after SLN analysis. The PBMC RT-PCR population, however, included patients with both histologically negative and positive SLNs.

Disease-free survival (DFS) was calculated from the date of random assignment to the date of the first recurrence. Distant–disease-free survival

Table 2. Clinical and Pathologic Characteristics of the Patient Population With Regard to PBMC RT-PCR Results

	PBMC RT-PCR Combined (positive RT-PCR, negative RT-PCR) (n = 820 patients)						
	Combined		Positive RT-PCR		Negative RT-PCR		
Characteristic	No.	%	No.	%	No.	%	*P**
Sex							.97
Male	452	55	63	56	389	56	
Female	360	44	50	44	310	44	
Unlisted	8	1					
Age, years							.66
Median	51						
Range	18-71						
Site of primary tumor							.66
Trunk	375	46	49	43	326	47	
Extremity	361	44	55	48	306	44	
Head or neck	79	10	10	9	69	10	
Other	0	0					
Unlisted	5	< 1					
Breslow thickness (mm)							.61
Median	1.7						
Mean	2.3						
Clark level							.65
I	1	< 1	0	0	1	< 1	
II	9	1	1	1	8	1	
III	173	21	25	23	148	22	
IV	580	78	78	71	502	74	
V	25	3	5	5	20	3	
Unknown	32	4					
Ulceration							.4
Present	223	27	31	27	192	27	
Absent	585	71	82	71	503	72	
NA	6	1	2	2	4	1	
Unknown	6	< 1					
SLN result by H and E and/or IHC							.006
Positive	208	25	41	36	167	24	
Negative	609	75	73	64	536	76	
Unknown	3	< 1					

Abbreviations: SLN, sentinel lymph node; RT-PCR, reverse transcriptase polymerase chain reaction; PBMC, peripheral blood mononuclear cells; NA, not available; H and E, hematoxylin and eosin; IHC, immunohistochemistry.

*For continuous variables, *P* value for testing for a difference in means for RT-PCR results. For categorical variables, *P* value for χ^2 test for association with RT-PCR results, omitting unknown categories. For the RT-PCR results, the *P* value compares the two proportions.

Table 3. RT-PCR Profile of the SLNs Collected for SLN-Negative Patients in the Sunbelt Melanoma Trial

Profile	No.	No. Positive	%
SLNs collected	3,505		
Median SLNs/patient	2		
Range SLNs/patient	1-10		
RT-PCR Marker			
Tyrosinase		999	28.5
MART1		891	25.4
MAGE3		446	12.7
GP100		339	9.7
Protocol definition (tyrosinase + one other marker)		874	24.9

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; SLN, sentinel lymph node.

(DDFS) was calculated from the date of random assignment until the date of first distant recurrence. Overall survival (OS) was calculated from the date of random assignment to the date of death. Survival distributions were estimated using Kaplan-Meier methods and the log-rank test was used to assess the statistical significance of differences in DFS, DDFS, and OS between groups. Because of relatively early follow-up, DFS and DDFS might be surrogates for changes in OS, or reflect differences in the pattern of recurrence. Therefore, all three measures were evaluated. For continuous variables, P values are for two-sample, equal variance t tests. For categoric variables, P values represent χ^2 test for association, omitting unknown categories. P values $< .05$ were considered significant, but since many statistical tests were done for this article, P values between .01 and .05 should be considered marginally significant. All analyses were performed with GraphPad Prism Software (GraphPad Software Inc, San Diego, CA) and JMP software (SAS Institute Inc, Cary, NC).

RESULTS

RT-PCR Analysis of SLNs

The Sunbelt Melanoma Trial was open for accrual from June 1997 through October 2003. The data analysis for this study includes follow-up data through April 2005. The median follow-up for this cohort was 30 months. The clinical and pathologic features of the patient population are listed in Tables 1 and 2. For this cohort, RT-PCR analysis was performed in 3,505 SLNs from 1,446 H and E/IHC-negative patients. Among patients with RT-PCR–positive SLN, the median of RT-PCR-positive SLN was 2 (range, 1 to 10). Table 3 lists this information and the SLN RT-PCR results for individual markers.

There were no differences in DFS (P = .61; Fig 1A), DDFS (P = .093; Fig 1B), or OS (P = .36; Fig 1C) between RT-PCR–negative and RT-PCR–positive patients. Similarly, there were no differences in DFS (P = .58; Fig 2A), DDFS (P = .052; Fig 2B), or OS (P = .15; Fig 2C) when analyzed by the number of markers expressed. In total, 63 (10.2%) SLN RT-PCR–positive patients and 91 (11.0%) SLN RT-PCR–negative patients have developed recurrent disease (P = .60). There were no differences in the patterns of recurrence (local, regional, distant) between PCR-negative and PCR-positive patients (for either SLN or PBMC).

Furthermore, using P = .05, there were no statistically significant differences in DFS, DDFS, or OS between patients with RT-PCR–positive or –negative SLN when analyzed by treatment received (observation, lymph node dissection, or lymph node dissection plus adjuvant interferon alfa-2b [data not shown]). In addition, there was no difference in DFS (P = .57), DDFS (P = .06), or OS (P = .47) for patients with multiple RT-PCR–positive SLNs when compared with those with 0 or 1 RT-PCR–positive SLN.

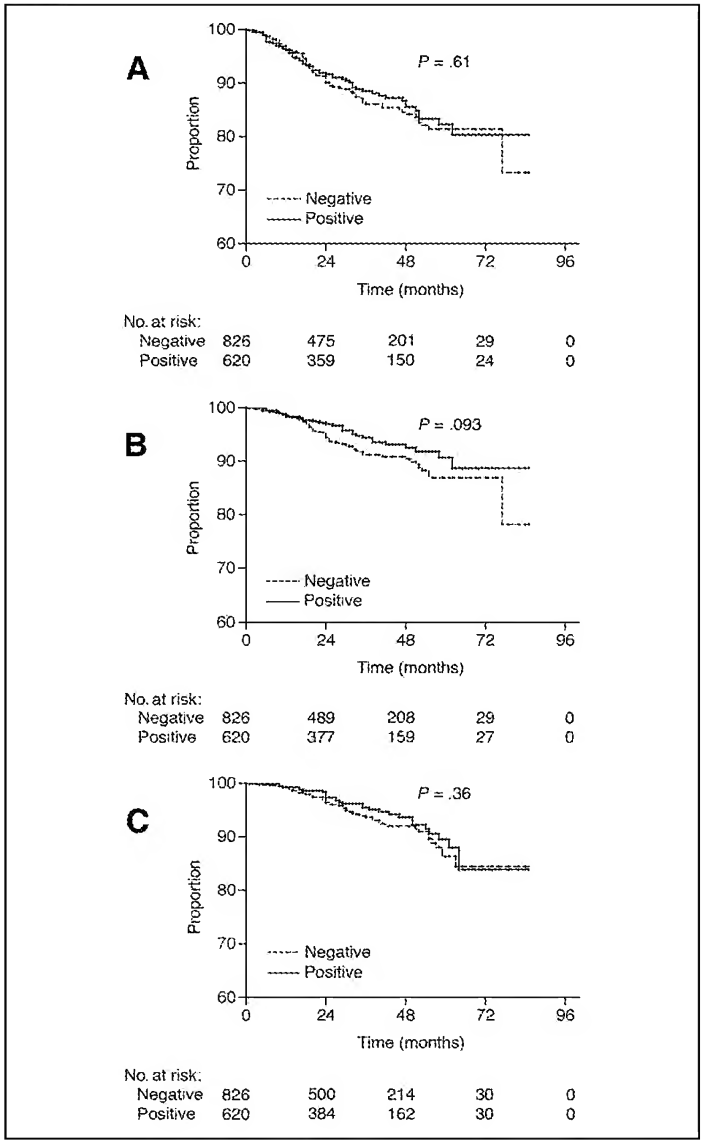

Fig 1. Kaplan-Meier survival analyses comparing patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative sentinel lymph nodes. (A) Disease-free survival (DFS); (B) distant-DFS (DDFS); (C) overall survival.

For SLNs, all prognostic factors in Table 1 were included in multivariate Cox proportional hazards models. After simultaneous adjustment for these known prognostic factors, RT-PCR (DFS, P = .23; DDFS, P = .14; OS, P = .41) was not significant in any of the Cox models. For blood RT-PCR (Table 2), similar models were considered. RT-PCR for PBMCs was likewise not significant for any survival end points (DFS, P = .89; DDFS, P = .10; OS, P = .07). A variable measuring the number of days from the first blood sample until a patient tested positive for blood RT-PCR or the last blood sample was drawn was added to the Cox models and found to be significant, but testing positive for RT-PCR still did not negatively influence any of the survival end points. Additional analysis was

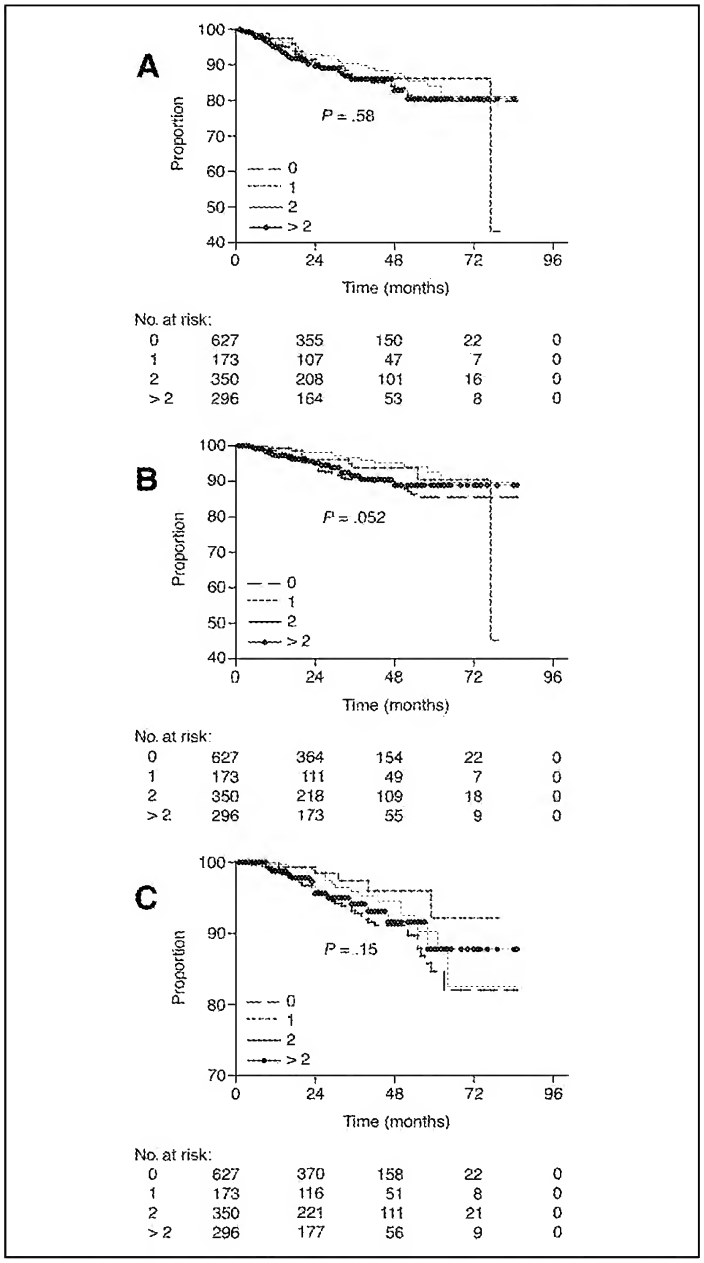

Fig 2. Kaplan-Meier survival analyses for patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative sentinel lymph nodes by the number of markers detected. (A) Disease-free survival (DFS); (B) distant–DFS (DDFS); (C) overall survival.

performed to determine, what if any, effect the number of positive markers had. For SLNs, there was no effect of positive marker count on DFS ($P = .7$) or OS ($P = .2$) even when considering factors known to influence outcome, such as sex, tumor site, thickness, and ulceration. However, there was a slight effect for males ($P = .022$) on DDFS. For the PBMC cohort, there were only 16 patients with more than one positive marker, making similar analyses of these data not useful.

The associations between characteristics in Tables 1 and 2 and the survival measures were investigated. It was found that for RT-PCR–negative patients, but not RT-PCR–positive patients, males generally had worse survival, and those males and females with head-neck melanomas had worse survival. As would be expected, ulceration adversely influenced all survival measures for both RT-PCR–positive and –negative patients. Clark level influenced survival minimally.

RT-PCR Analysis of PBMCs

RT-PCR analysis was performed on PBMC samples from 820 patients. The median follow-up for this cohort was 37 months. Considering patients included in the PBMC RT-PCR analysis, 25.4% had histologically positive SLNs, with a mean of 1.6 total positive nodes per patient. Among the 820 patients who were observed by serial RT-PCR analysis of PBMCs, 115 (14%) had evidence of at least one RT-PCR marker at some point during follow-up. Analysis of baseline blood samples revealed that there were no differences in DFS ($P = .86$; Fig 3A), DDFS ($P = .24$; Fig 3B), or OS ($P = .07$; Fig 3C) between patients with a detection of at least one PBMC RT-PCR marker at any time during follow-up and those who never had a marker detected. Analysis of PBMC RT-PCR by the number of markers expressed revealed that the DFS ($P = .006$; Fig 4A) and DDFS ($P = .03$; Fig 4B)

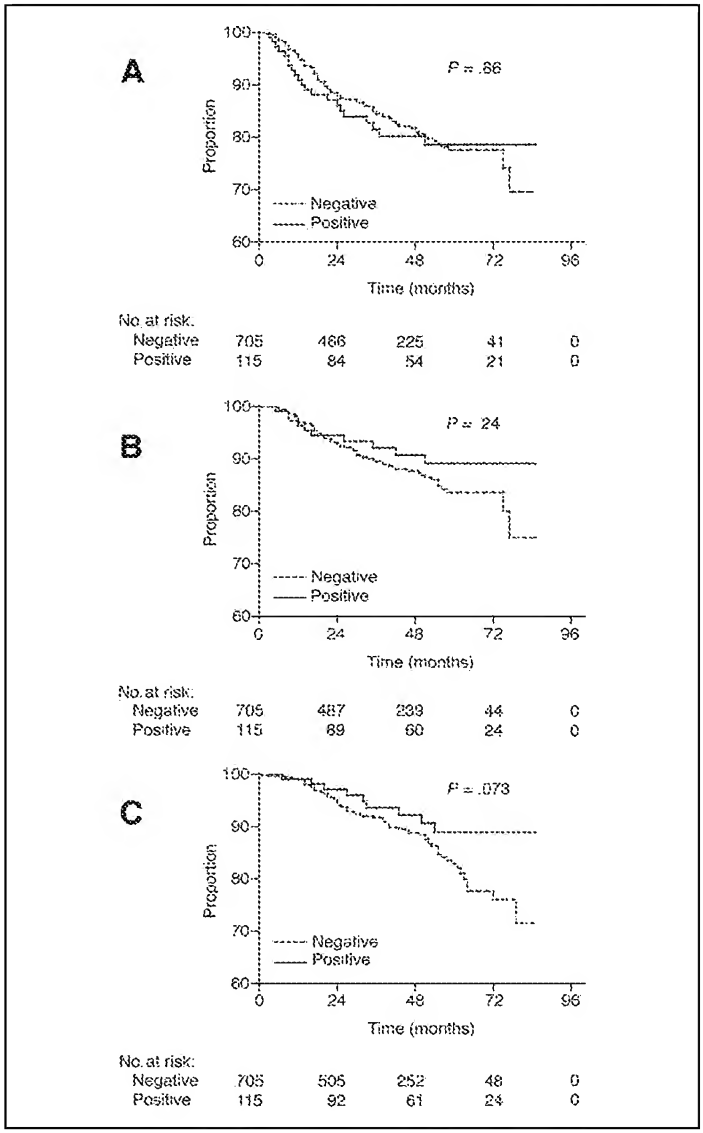

Fig 3. Kaplan-Meier survival analyses comparing patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative peripheral blood mononuclear cells. (A) Disease-free survival (DFS); (B) distant–DFS (DDFS); (C) overall survival.

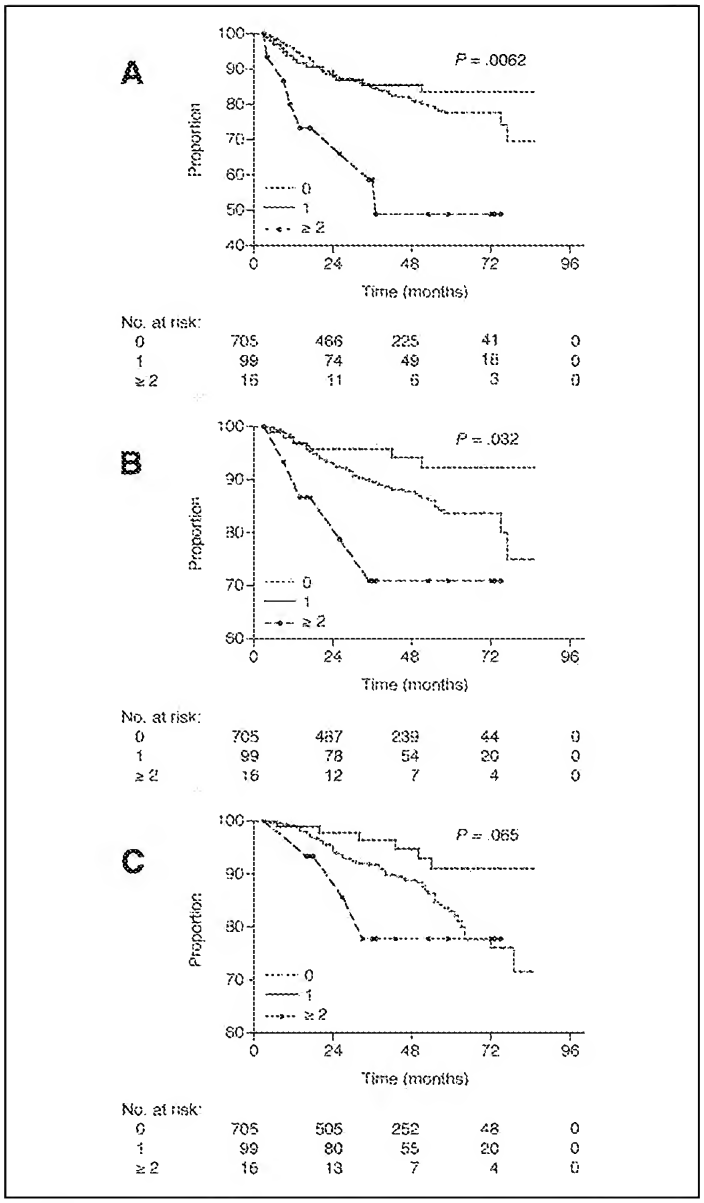

Fig 4. Kaplan-Meier survival analyses for patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative peripheral blood mononuclear cells by the number of markers detected. (A) Disease-free survival (DFS); (B) distant–DFS (DDFS); (C) overall survival.

were worse for patients with more than one marker detected at any point during follow-up; OS approached but did not reach statistical significance ($P = .065$; Fig 4C). There were no significant differences in DFS, DDFS, or OS when PBMC results were analyzed by individual markers (data not shown). Alternatively, when analyzing the positive markers that were not tyrosinase as an explanatory variable, and incorporating into proportional hazards models, these variables are not significant for DFS, LRR, or DDFS in univariate or multivariate models. Similarly, there were no significant differences in DDFS or OS (data not shown) between patients with positive or negative PBMC RT-PCR results when analyzed by the treatment received, or when analyzed by histologically node-negative or node-positive patients. There was a difference noted in DFS ($P = .03$) between RT-PCR–negative and –positive patients who received no additional treatment, although this appears to be a clinically insignificant phenomenon.

DISCUSSION

SLN status is the strongest predictor of survival for early-stage melanoma.[9,30-33] The burden of disease within the node appears to affect outcome as well,[34] thus providing additional impetus to identify patients with micrometastatic disease who may be candidates for additional therapy. In our study, which is the largest study of molecular staging of melanoma to be reported, we found no clinically significant benefit for RT-PCR analysis. Others have observed similar results,[21] and concluded that RT-PCR does not give prognostic information above that provided by thorough SLN histopathology.

However, other smaller studies have demonstrated varying degrees of predictive value for RT-PCR testing of SLNs.[12,18-20,22,35-37] Shivers et al[35] found that PCR for tyrosinase predicted recurrence and overall survival. Interestingly, there was only one recurrence of 44 (2%) histologically negative, PCR-negative SLN patients and six of 47 (13%) recurrences in the patients whose SLNs were histologically negative and PCR positive.[35] Li et al[38] have shown that detection of tyrosinase mRNA in SLNs correlates with histological predictors of adverse outcome. In addition, in a study of 129 patients, Gradilone et al[36] demonstrated that expression of PCR markers tyrosinase and/or MIA strongly correlates with DFS.

The specificity of tyrosinase has been questioned, mainly because of the possibility of a false-positive result.[39] This has led investigators to evaluate multiple-marker RT-PCR to improve specificity.[14,18,20] Using a multiple-marker RT-PCR assay (MART1, MAGE3, GalNAc-Tβ, and Pax3), Takeuchi et al[40] found prognostic value for SLN RT-PCR. In a much smaller, retrospective study, Bostick et al[18] were able to correlate multiple-marker PCR (tyrosinase, MART1, MAGE3), and histological evidence of SLN metastasis with increased recurrence. Others have reported similar results in small studies.[15,22,38]

Despite a multimarker RT-PCR assay, sensitive Southern blot detection, and analysis at a central laboratory, we were not able to demonstrate prognostic significance for RT-PCR. Experimental and procedural variables that may affect RT-PCR include specimen processing, RNA condition, choice of primers, and RT-PCR conditions. Because the study began in 1997, the assay was not based on semiquantitative real-time RT-PCR, which would be the present standard. Perhaps a more quantitative assessment of RT-PCR markers would result in a more predictive test. The fact that the assay had no false-positive results in 100 nonmelanoma patient lymph nodes suggests that indiscriminate amplification of nonspecific mRNA was not likely the main problem.[29] mRNA was detected, but low levels of expression may be clinically insignificant. It is possible that a small number of metastatic melanoma cells within a node do not establish themselves as clinically significant metastases, and/or that the patient's own defenses eliminate these cells. In addition, the fact that 43% of patients with H and E/IHC-negative SLNs had a positive RT-PCR test, which greatly overestimates the fraction of patients likely to experience recurrence, suggests that greater assay specificity is needed. Perhaps other markers would have provided different results; however, 75% of the 647 histologically positive SLNs were positive for tyrosinase. This percentage is similar to previous reports.[18,21,41,42]

Another consideration is the fact that 11% of the H and E/IHC- and PCR-negative patients have experienced recurrence. This might be explained by sampling error, given that ≤ 2 mm^3 of each SLN was tested. The portion of the SLN analyzed by PCR simply may not have contained micrometastases, although this is nearly identical to the percentage of H and E/IHC- and PCR-positive patients (10.2%) who have developed recurrent disease. Any sampling method is just that—a sampling—and even with serial sectioning and IHC used in this study, only a small portion of each node was tested histologically or by RT-PCR. Whether sampling a larger portion of each node would have resulted in different results is uncertain, yet the fact that more than 40% of patients had RT-PCR evidence of melanoma cells in the SLN argues against the likelihood that a larger sample would have resulted in clinically applicable data.

Another potential method of molecular staging of melanoma is detection of metastatic melanoma in blood. In the early 1990s, Smith et al[16] demonstrated the feasibility of RT-PCR for melanoma in peripheral blood. Subsequently, several studies have suggested that RT-PCR evidence of circulating melanoma cells may have prognostic significance, although the results are somewhat mixed.[12,17,28,43-48]

In this study, RT-PCR analysis of PBMCs demonstrated that the prognosis of patients with one marker was no different than for those who had no markers detected. Expression of more than one marker was associated with worse DFS and DDFS; OS was not different. However, only 2% of patients had evidence of more than one PBMC RT-PCR marker, which underestimates the fraction of patients that will experience recurrence. Thus, the PBMC RT-PCR assay, using more than one marker detected as the definition of a positive test, is not likely to have sufficient sensitivity to make it clinically useful. Because the study did not randomly assign patients based on PBMC RT-PCR results, the protocol did not include an a priori definition of a positive PBMC RT-PCR result: the PBMC results were observational. The goal of the PBMC studies was to provide a definition of a positive test based on analysis of these data. Five milliliters of blood may not have been sufficient to capture a limited number of circulating tumor cells, although this volume is similar to those used by other investigators.[46-49] Finally, although individual sites processed the blood samples, a central laboratory performed the PCR testing. The central laboratory provided the processing kits, which included a simple, step-by-step procedure designed to reduce variability, and although significant variability is unlikely, it is possible.

Another caveat is the relatively short median follow-up for this study (30 months). Kammula et al[21] found initial differences in survival for patients with RT-PCR–positive versus –negative SLNs that disappeared with longer follow-up, and recommended that such studies should include long-term follow-up. In our study, we found the same phenomenon: initial results with limited follow-up (12 months) demonstrated divergence of the disease-free survival curves, which subsequently converged. Given the size and statistical power of our study, it is unlikely that additional follow-up will result in a significant divergence of the curves.

In conclusion, using a large, multicenter, randomized, prospective study, we did not find any predictive value of SLN RT-PCR analysis. Detection of more than one marker in PBMC was associated with worse prognosis, although the clinical utility of this assay is likely limited. Additional refinements in molecular staging of melanoma may hold promise for the future. However, we were not able to demonstrate that molecular staging of melanoma would provide significant prognostic information above and beyond standard SLN histopathology. At present, RT-PCR for molecular staging of melanoma should be considered investigational, and should not be used to direct therapy.

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Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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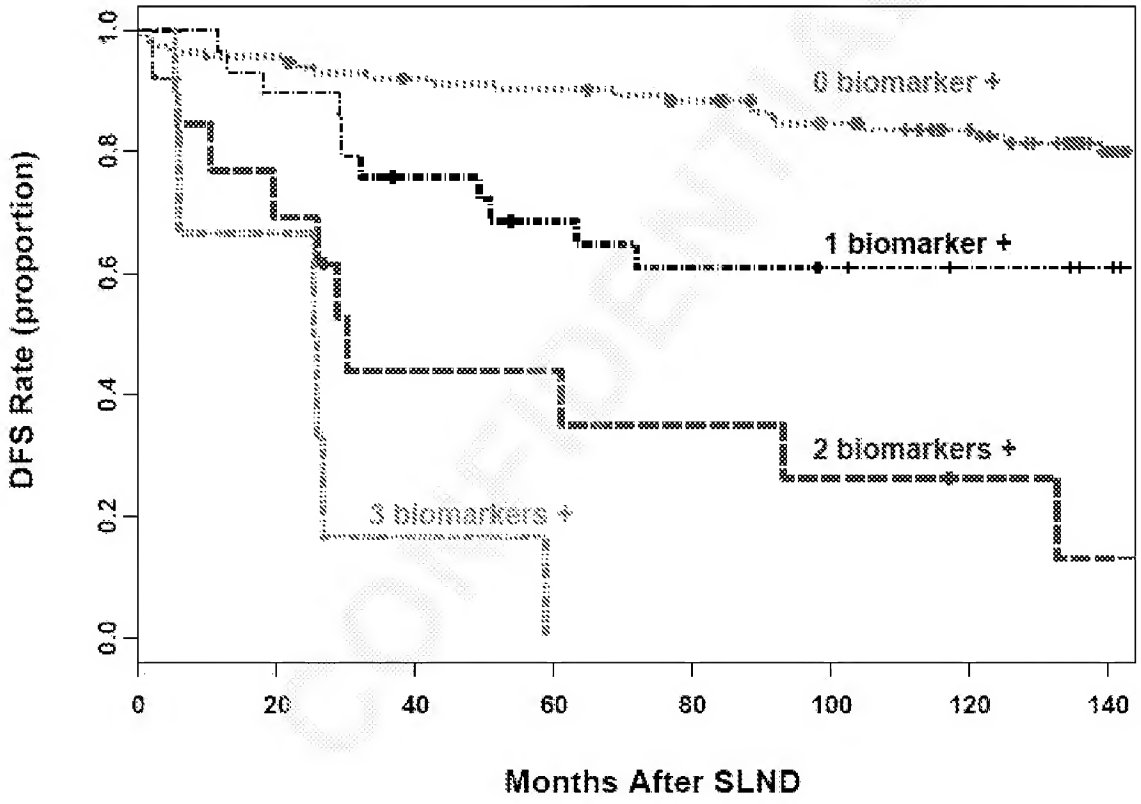

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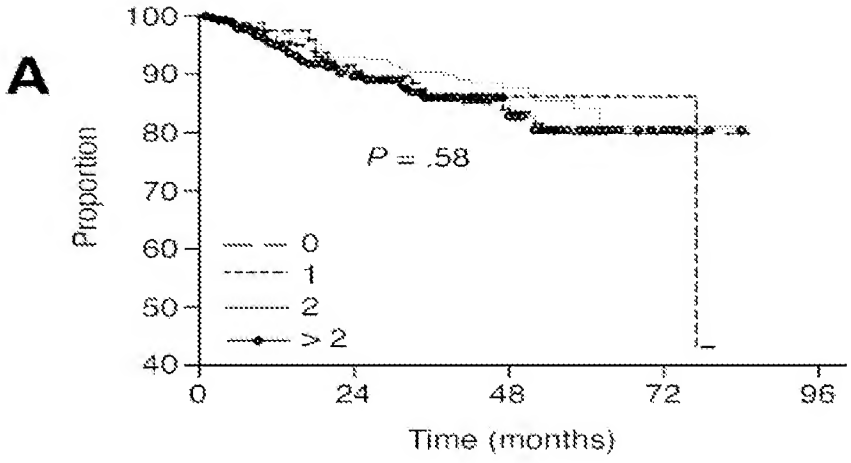

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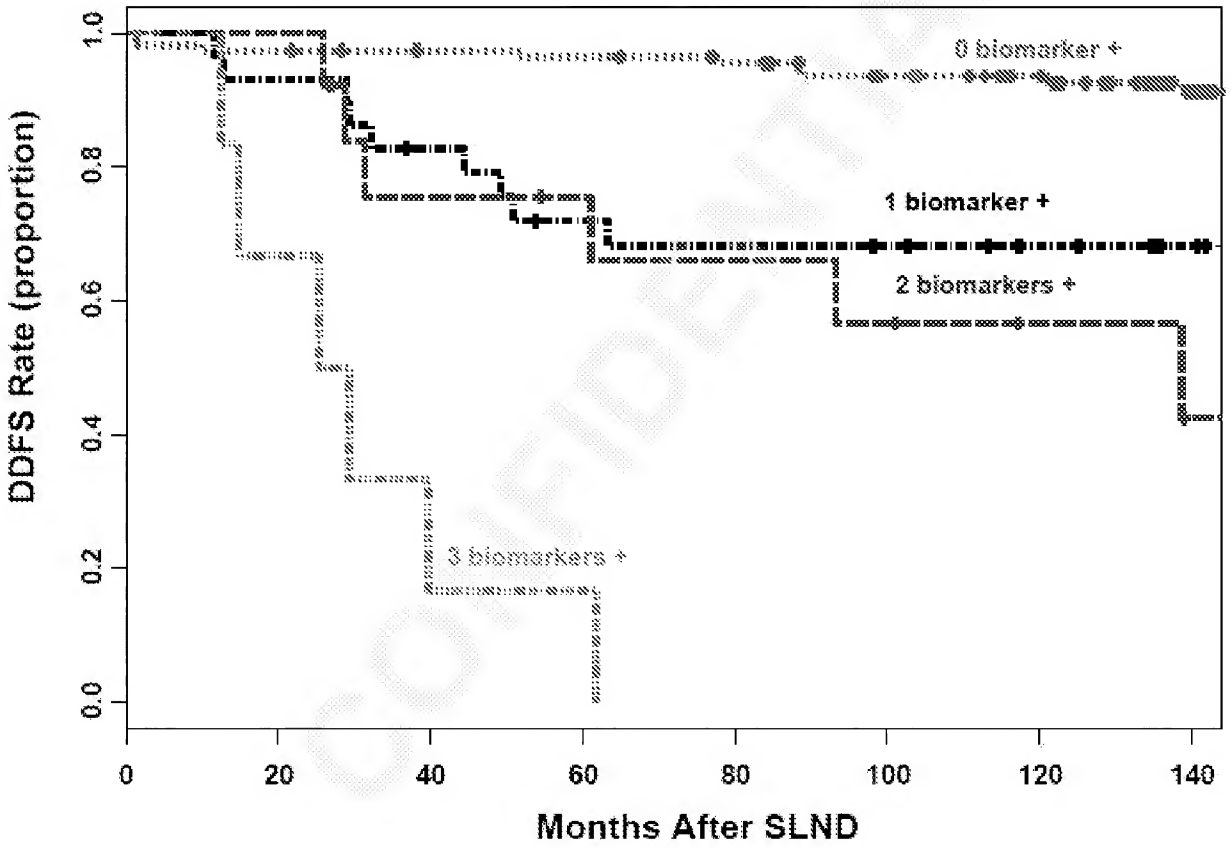

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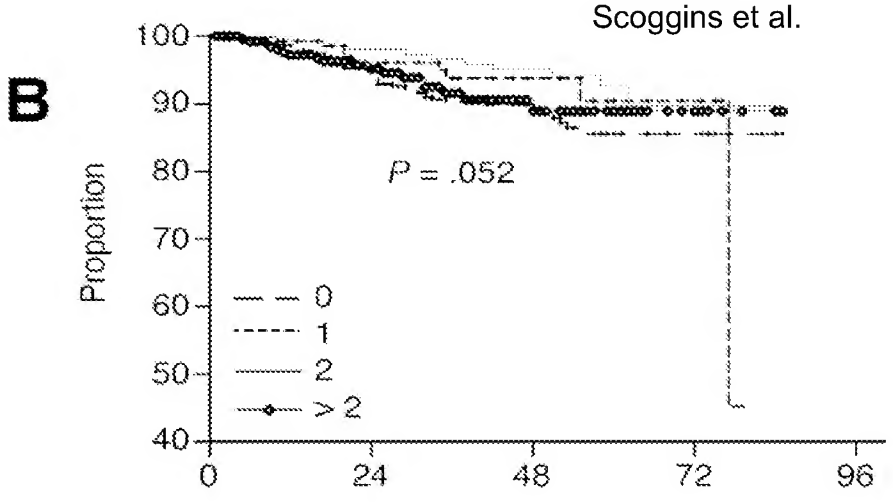

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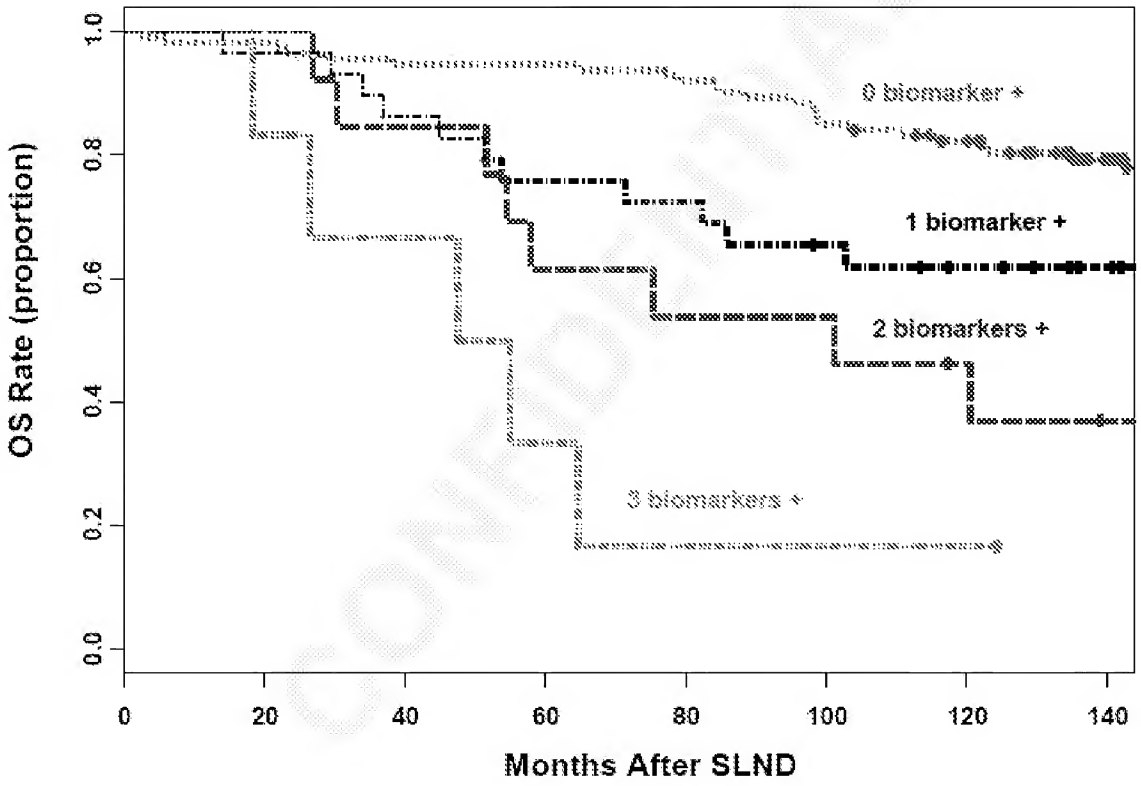

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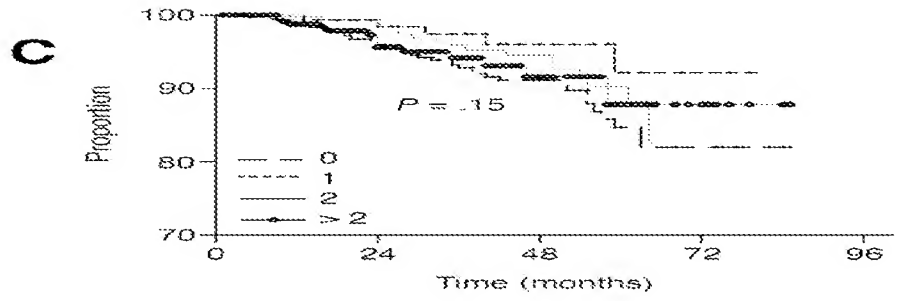

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